U.S. GEOLOGICAL SURVEY
NATIONAL WATER QUALITY LABORATORY
SOP — Laboratory Analytical Method or Procedure

SOP #	EFFECTIVE DATE:	QUANTITATIVE FIXED-COUNT METHOD FOR PROCESSING BENTHIC MACROINVERTEBRATE SAMPLES
BS0333.0	April 7, 2000	
[X] New [ ] Revision	PAGE 1 of 13	
WRITTEN BY: S.R. Moulton, II and S.A. Grotheer	APPROVED BY: Merle W. Shockey	

1. Scope, Application and Summary

1.1. The goal of the quantitative fixed-count processing method (Appendix 1) is to sort, identify, enumerate, and estimate total relative abundance of benthic macroinvertebrate (BMI) taxa from a sample. The fixed-count method can be applied to BMI samples collected in the field using quantitative, semiquantitative, or qualitative sampling methods. The quantitative fixed-count method is based on a minimum number of organisms sorted from the sample, and is defined by a project's data-quality objectives (for example, 100-, 200-, or 300-organism fixed-count target). Samples with more organisms than the fixed-count target are subsampled using a subsampling frame partitioned into 5.1 cm x 5.1 cm grids (Moulton and others, 2000). Multiple, randomly selected portions of the original sample (stage-1 grids) are selected and the average number of organisms in each stage-1 grid is determined. Using the average number of organisms in each stage-1 grid, an appropriate subsampling strategy is determined. Grids are randomly selected and sorted. Depending on the fixed-count target, total sorting time using this method is limited to a maximum of 8 hours. Large-rare organisms are sorted from any remaining unsorted portions of the sample. Organisms are identified and enumerated.

1.2. Lab codes supported by this method—2172, 2174, 2175

1.3. Reporting units and levels—Standard Taxonomic Assessment and Rapid Taxonomic Assessment (see SOP No. BS0334.0)

1.4. Detection limits—not applicable

1.5. Interferences

1.5.1. Sorting effectiveness varies with the type and amount of sample detritus. Excessive organic detritus makes it difficult to distinguish organisms (especially small, cryptic organisms) from the sample matrix.

1.5.2. Sample volumes in excess of 750 mL are difficult to process with this method because it is difficult to achieve a thin even distribution of sample in the subsampling frame; the volume must be reduced to less than 750 mL before using the fixed-count method.

1.5.3. Substantial amounts of inorganic material (for example, sand) or large organic debris (for example, sticks and leaves) inhibit the uniform distribution of a sample in a subsampling frame. Such debris should be removed before proceeding with the method (see SOP BS0331.0).

1.5.4. Substantial amounts of filamentous algae should be uniformly distributed in the subsampling frame as best as possible. Clumps of algal filaments must be separated carefully, and delicate organisms (for example, mayfly larvae) must be handled gently to minimize damage or loss of taxonomically valuable body parts such as gills and legs.

2. Reasons for Revision and Summary of Changes: This is a new SOP.

3. Health and Safety Warnings

3.1. Personal Safety

3.1.1. Wear long pants and closed-toed shoes at all times when working in the laboratory.

3.1.2. Wear an apron, rubber gloves, and protective eyewear during sample preparation.

3.1.3. Know the location of the nearest eyewash and shower stations.

3.1.4. Do not eat or drink in the laboratory.

3.1.5. Follow other safety procedures described in the USGS Occupational Hazards and Safety Procedures Handbook (September 1999).

3.2. Chemical Safety

3.2.1. Only work in the laboratory when the room ventilation system and fume hoods are working properly. Leave the laboratory and contact the BG supervisor if the ventilation systems are not working properly.

3.2.2. Use the preservative waste pump system to transfer preservative waste from the fume hood to the storage barrel. Contact the BG Supervisor if the system is not functioning properly. Contact the BG Safety Committee representative when the storage barrel is full and needs to be replaced.

3.2.3. Know the location of and be familiar with the Material Safety Data Sheets (MSDS) for each chemical used in the laboratory.

3.2.4. Know how to report and handle chemical and sample spills using procedures described in the NWQL Chemical Hygiene Plan (available from the Safety Program).

3.2.5. Follow other standard safety guidelines as describe in National Research Council (1995).

4. Sample Preservation, Handling, Containers, Analytical Processing/Holding Times, Cautions and Disposal

4.1. Each unprocessed sample is stored in a wide-mouth Nalgene™ bottle (≤ 1 L) provided by the customer. Unprocessed samples are kept in ventilated metal cabinets for each project in the BG storage facility.

4.2. Each sample received in formalin should be washed and re-preserved in 70-percent ethanol within 2 weeks of receipt at the NWQL. Samples preserved in ethanol can be stored indefinitely.

4.3. Each portion of the processed sample is archived and placed in a ventilated metal cabinet located in the BG storage facility.

4.3.1. Sorted Sample Remnant

4.3.1.1. Place in a wide-mouth glass container.

4.3.1.2. Fill container with enough 70-percent ethanol to cover the remnant; secure the lid.

4.3.1.3. Place a label on the outside of the sample container with the sample ID, the name of the individual who sorted the sample, and the date the sample was sorted.

4.3.1.4. Place the sample remnant and vials of unidentified sorted BMIs in the QC cabinet.

4.3.2. Unsorted Sample Remnant (minus large-rare organisms)

4.3.2.1. Return to original sample container.

4.3.2.2. Fill the container with 70-percent ethanol to cover the remnant; secure the lid.

4.3.2.3. Place a label on the outside of the sample container with the name of the individual who sorted the sample original, and the date the sample was sorted.

4.3.2.4. Return the sample container to the cabinet in the BG storage facility where other samples for the project are kept.

4.3.3. Sorted BMIs

4.3.3.1. Place in polyseal screw-cap vials.

4.3.3.2. Fill the vial with 70-percent ethanol and secure the cap.

4.3.3.3. Place a label in each vial with the sample ID, the portion of sample (either lab large-rare or grids), and the name of the individual who sorted the sample.

4.3.3.4. After sorting is complete, place vials on a shelf in the QC cabinet. The sample is ready for the sorting effectiveness check.

4.3.3.5. Following the sorting effectiveness check, BMI vials are placed in the taxonomists' cabinet for identification.

4.3.4. Identified BMIs

4.3.4.1. Place in polyseal screw-cap vials.

4.3.4.2. Fill the vial with 70-percent ethanol and secure the cap.

4.3.4.3. Place a label in each vial with at least the sample ID, the scientific name or the organism, the name of taxonomist performing the identification, and the year the identification was made.

4.3.4.4. After all organisms from a sample have been identified, place vials in the QC cabinet on a shelf with all other samples that have been completed in a given week.

4.3.4.5. Following QC, selected vials of identified BMI taxa will be added to the reference collection by a QC representative. All non-referenced vials are returned to the appropriate cabinet in the BG storage facility.

4.4. Each sample remnant is disposed according to NWQL Technical Memorandum 00.03.

4.5. Work order/worksheet handling—not applicable

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5. Preparation of Reagents/Standards/Solvents

5.1. The following chemicals are used to process samples with this method:

5.1.1. Tap water

5.1.2. 70-percent ethanol

6. Apparatus

6.1. Labware

6.1.1. Forceps

6.1.2. Scoopula® or other small scoop

6.1.3. Fine-bristle brush

6.1.4. Polyseal screw-cap vials

6.1.5. Sorting labels

6.1.6. Vial racks

6.1.7. Scissors

6.1.8. White sorting trays of various sizes (for example, 15 x 20 cm and 20 x 30 cm)

6.1.9. Plastic wash basins

6.1.10. Scrub brush

6.1.11. Wash bottles

6.1.12. Computer-generated random number tables

6.1.13. Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet

6.2. Equipment

6.2.1. Tally counter

6.2.2. Dissecting microscope

6.2.3. Light source (fiber-optic illuminator or portable incandescent lamp)

6.2.4. Standard metal sieve (mesh size equal to field-collection mesh size)

6.2.5. Post-sort sieve (210 µm mesh)

6.2.6. Subsampling frames (12-, 24-, and 42-grid frames, each with 5.1-cm x 5.1-cm grids)

6.2.7. Estimation trays (49- and 81-grid frames, each with 1.3-cm x 1.3-cm grids)

7. Analysis

7.1. Prepare the sample according to SOP No. BS0331.0.

7.2. Ensure that any substantial inorganic material of the sample has been removed.

7.3. Select a stage-1 subsampling frame in which the sample can be distributed. The approximated sample volume is used to determine which frame is selected.

Sample volume (mL)	Suggested subsampling frame
< 250	12 grid
250 – 500	24 grid
500 – 750	42 grid

7.4. If the sample volume is substantially less than 250 ml, the sample can be adequately dispersed in an 81-grid estimation tray. If organism density appears to be low, proceed with a rapid estimation of organism density in the sample.

7.4.1. Distribute sample in an 81-grid estimation tray.

7.4.2. Count the organisms in eight randomly selected estimation tray grids.

7.4.3. Multiply the total number of organisms in the eight grids by 10 to estimate the total number of organisms in the sample.

7.4.4. If the estimated total number of organisms does not exceed the target by greater than 50 percent, sort the entire sample. For example, if the target is 300 organisms, the rapid estimate should not exceed 450 organisms.

7.4.5. If the estimated total number of organisms exceeds the target by greater than 50 percent, then select a 12-grid subsampling frame.

7.5. Place the selected stage-1 subsampling frame in a large, flat-bottomed tray.

7.6. Transfer the sample into the stage-1 subsampling frame

7.6.1. Distribute the sample uniformly over the entire subsampling frame.

7.6.2. Fill the subsampling frame with just enough water to cover the sample. Too much water will cause some organisms and debris to float and adversely affect the distribution of the sample.

7.6.3. Ensure that larger debris such as leaves and sticks are removed and do not inhibit a uniform sample distribution.

7.6.4. Remove any air bubbles trapped beneath the mesh on the subsampling frame.

7.6.5. If necessary, re-distribute the sample as uniformly as possible over the subsampling frame.

7.6.6. Allow the sample to settle for a few seconds.

7.6.7. Keeping the subsampling frame level, carefully lift it out of the water and drain.

7.6.8. Set the subsampling frame aside, and discard the water in the large flat-bottomed tray.

7.6.9. Return the subsampling frame to the flat-bottomed tray, and cover it until the grids are removed.

7.6.10. Mist the sample with water periodically to prevent drying.

7.7. Randomly select five stage-1 grids from the stage-1 subsampling frame.

7.7.1. Select five pairs of random numbers to represent row/column coordinates on the stage-1 subsampling frame. Record the coordinates on the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet.

7.7.2. Use the coordinates to locate the five grids in the stage-1 subsampling frame.

7.7.3. Separately remove the sample from each stage-1 grid and place it in a jar numbered to correspond to the coordinate of the grid on the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet. Use a scoop to remove most of the material; forceps or a fine-bristle paintbrush may be helpful in removing small organisms and residual detritus.

7.8. Estimate the mean number of organisms for each of the five stage-1 grids.

7.8.1. Select either a 49-grid or 81-grid estimation tray on the basis of the stage-1 grid volumes; the same-size estimation tray should be used for all stage-1 grids.

7.8.2. Select three pairs of random numbers for each stage-1 grid to represent row/column coordinates in the estimation tray. Record the coordinates on the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet.

7.8.3. Distribute the sample for each stage-1 grid as thinly and evenly as possible in the estimation tray.

7.8.4. Locate the estimation-tray grid corresponding to the first pair of coordinates and count all organisms contained in the grid.

7.8.4.1. If an organism lies across two or more grids, count it if its head lies within the selected grid.

7.8.4.2. If an organism lies across two or more grids and its head does not lie within the selected grid, then only enumerate the organism if its head does not lie in another selected grid.

7.8.5. Record the number of organisms in each estimation tray grid on the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet.

7.8.6. Return the stage-1 grid (sample and tallied organisms) to its corresponding numbered jar.

7.9. Enter the sample information from the completed estimation into the Microsoft® Excel version of the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet corresponding to the chosen fixed-count target to obtain a recommended subsampling strategy. Available fixed-count worksheets include:

500 MINIMUM COUNT.xls,

300 MINIMUM COUNT.xls, and

100 MINIMUM COUNT.xls.

7.9.1. Enter sample the sample identification code, the size of the stage-1 subsampling frame (12, 24, or 42), and the size of the estimation tray used (49 or 81).

7.9.2. Enter the three estimation tray grid counts for each stage-1 grid into the corresponding fields of the Microsoft® Excel version of the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet. (Note: the spreadsheet will perform all calculations to obtain the average number of organisms in each stage-1 grid.)

7.9.3. After the calculations are made, processing guidance is displayed in the electronic version of the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet. Transcribe the processing guidance onto the hardcopy of the worksheet.

7.9.4. Save the estimation results with the file name "*sampleID*.xls" on the QC Officers' computer.

7.10. Perform additional subsampling as recommended by the Microsoft® Excel version of the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet.

7.11. If the Microsoft® Excel version of the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet is not available, select an appropriate subsampling strategy using the guidance presented in Moulton and others (2000).

7.12. Consult the QC Officer if in doubt how to proceed.

7.13. Procedures when 2-stage subsampling is recommended.

7.13.1. Record the stage-2 subsampling-frame-size on the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet.

7.13.2. Randomly select and process the appropriate number of stage-2 grids from the stage-2 subsampling frame in the same fashion they were selected from the stage-1 subsampling frame. Record the grid coordinates in the lower section of the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet.

7.13.3. Separately remove the sample from each stage-2 grid and place in a jar numbered to correspond to the coordinates of the stage-2 grid.

7.14. Place empty vials in a rack labeled with the following taxonomic groupings: Gastropoda, Bivalvia, Oligochaeta, Hirudinea, Hydrachnidia, Decapoda, Amphipoda/Isopoda, Ephemeroptera, Odonata, Plecoptera, Heteroptera, Megaloptera, Trichoptera, Lepidoptera, Coleoptera, Diptera (excluding Chironomidae), Chironomidae, Other BMIs.

7.15. Sort organisms from the randomly selected grids.

7.15.1. Record the time when sorting begins on the data sheet.

7.15.2. Place each grid or a portion of the grid in a petri dish or estimation tray. Grid marks on the petri dish or estimation tray can be used as a guide while sorting so parts of the sample are not missed.

7.15.3. Begin sorting organisms from the petri dish or tray under a dissecting microscope at 10X magnification.

7.15.3.1. Do not sort the following organisms or life stages: vertebrates, arthropod exuviae, empty mollusk shells, branchiobdellids (worm-like crayfish parasites), eggs, microcrustaceans, or terrestrial organisms. If in doubt, sort the organism but do not count it.

7.15.3.2. Do not pry open debris to search for burrowing organisms.

7.15.4. Place organisms into vials by taxonomic grouping.

7.15.5. Use a counter to keep track of the number of organisms sorted from each grid. Upon completion, record the coordinates of the grid, the number of organisms sorted, and the sort time in the appropriate space in the lower section of the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet.

7.15.6. Always sort a grid to completion.

7.15.7. If it appears that the target will be exceeded by more than 50 percent after completely sorting either the first or second grid, consult the QC Officer for guidance.

7.15.8. Always sort at least three grids.

7.15.9. When to stop sorting additional grids:

7.15.9.1. if the numeric target is reached at the completion of a grid.

7.15.9.2. if the time limit for a given target is reached at the completion of a grid.

8 hours for a 300-organism target

3 hours for a 100-organism target

7.15.9.3. if sorting one more grid will cause the time limit to be exceeded, even if the target has not been reached.

7.16. Record all final subsampling information on the worksheet.

7.17. Obtain the initials of the QC Officer or his/her representative, indicating that the subsampling strategy and the information on the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet correspond.

7.18. Perform a 15-minute visual-sort for large-rare organisms occurring in any unsorted sample detritus. This may include smaller, more abundant organisms that were not represented in the grid subsamples.

7.19. Place all sorted large-rare organisms, regardless of taxonomic grouping, in a separate, labeled container; preserve with 70-percent ethanol.

7.20. Clean-Up

7.20.1. Combine all sorted grid remnants and place in a single appropriately sized container.

7.20.2. Place all sample components in labeled containers. Store all components for a sample together; place all samples for a project together in the laboratory storage space designated for each taxonomist.

7.20.3. Rinse and clean all subsampling frames, estimation trays, sieves, and wash basins used to process the sample.

7.20.4. Scrub sieves with a brush and rinse from both sides to remove entrained sample debris.

7.20.5. Wipe up water and clean workstation. Put supplies and equipment away.

7.21. Enter the final subsampling information into the Microsoft® Excel version of the worksheet.

7.22. Print a hardcopy of the Microsoft® Excel version of the completed worksheet.

7.23. Identify sorted organisms to project-specific taxonomic levels using the method described in SOP No. BS0335.0.

8. Quality Control and Quality Assurance

8.1. Inspect all sieves, subsampling frames, and estimation trays before and after processing. To ensure there are no attached organisms or remaining sample debris.

8.2. Verify that information recorded on the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet is correct prior to the large-rare sort. A second taxonomist performs this verification.

8.3. Determine Sorting Effectiveness

8.3.1. The sorting effectiveness check detects gross errors. Typical errors include incompletely sorting one or more grids or routinely missing certain taxa (for example, tiny case-building caddisflies) because the sorter is unaware of them in the sample.

8.3.2. A QC representative performs re-sorting on each sample prior to taxonomic identification.

8.3.3. Samples sorted by personnel inexperienced in the processing methodology are re-sorted one grid at a time while the sample is being processed. At least the first five samples processed are reviewed by a QC representative in this manner. If performance is satisfactory, routine sorting effectiveness checks are performed.

8.3.4. Routine Sorting Effectiveness

8.3.4.1. Each sorted sample remnant is re-sorted at 10X magnification.

8.3.4.2. Re-sort time is limited to 10 percent of the original sort time. For example, if a sample were originally sorted in 180 minutes, then the re-sort is limited to 18 minutes.

8.3.4.3. Organisms obtained during a re-sort are counted and added to the appropriate sorting vials.

8.3.4.4. The total number of organisms is recorded on the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet.

8.3.4.5. The sorting effectiveness statistic (E_s) is calculated as:

$$E_S\,(\%) = 100\% \cdot \frac{S}{R+S}$$

where, R = the total organisms obtained during the resort of the grid remnants

S = the total organisms originally obtained from the sorted grids

E_s should be ≥ 80 percent.

8.4. Corrective Actions

8.4.1. Recommendations made by the QC representative are implemented in the sorting of future samples.

9. Data Acquisition, Calculations, and Data Evaluation/Reduction

9.1. Calculation of the laboratory subsampling correction factor [W = total grids in the stage-1 subsampling frame, X = total grids sorted from the stage-1 subsampling frame, Y = total grids in the stage-2 subsampling frame, Z = total number of grids sorted from the stage-2 subsampling frame]

	Subsampling strategy	
	1-Stage subsampling	**2-Stage subsampling**
Laboratory subsampling correction factor (L)	$L = \frac{W}{X}$	$L = \frac{W}{X^{1}} \times \frac{Y}{Z}$

[1]In 2-stage subsampling, X will typically be 5.

10. Data Management and Records Management

10.1. The completed hand-written Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet is filed with the remaining sample paperwork.

10.2. The Microsoft® Excel version of the Fixed-Count Processing—Subsampling and Preliminary Enumeration Worksheet is saved in the QC Officers' computer and backed-up regularly. A hardcopy is filed with the remaining sample paperwork.

10.3. Enter name of sorter, date sorting was completed, preparation time, sort time and the subsampling correction factor code (X:W or X·Z:W·Y, as defined above).

10.3.1. Example 1: If four stage-1 grids are sorted from a 24-grid, stage-1 subsampling frame, enter *4:24* in appropriate fields on the bench data sheet.

10.3.2. Example 2: If five stage-1 grids are combined and transferred from a 24-grid, stage-1 subsampling frame into a 12-grid, stage-2 subsampling frame, and four stage-2 grids are sorted, enter *20:288* in appropriate fields on the bench data sheet.

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11. Definitions

11.1. Estimation tray—a small gridded tray (either 49- or 81-grids) used to estimate the number of organisms in a stage-1 grid.

11.2. 1-stage subsampling—A procedure to obtain randomly selected square-grid subsamples from the original sample.

11.3. Stage-1 subsampling frame—gridded subsampling frame (either 12-, 24-, or 42-grids) used to obtain square-grid subsamples from the original sample.

11.4. Stage-1 grid—a randomly selected square-grid from a stage-1 subsampling frame.

11.5. Stage-1 subsample—the resulting composite of all sorted stage-1 grids.

11.6. 2-stage subsampling—A procedure to obtain randomly selected square-grid subsamples from a stage-1 subsample.

11.7. Stage-2 subsampling frame—gridded subsampling frame (either 12-, 24-, or 42-grids) used to obtain square-grid subsamples from a stage-1 subsample.

11.8. Stage-2 grid—a randomly selected square- grid from a stage-2 subsampling frame.

11.9. Stage-2 subsample—the resulting composite of all sorted stage-2 grids.

11.10. Visual sort—Sorting organisms from a sample without using any magnification.

11.11. Large-rare—large and generally “rare organisms” (occurring at low frequencies) present in a sample that may or may not be included in the sorted portion of a subsample.

12. References

12.1. Moulton, S.R., II, Carter, J.L., Grotheer, S.A., Cuffney, T.F., and Short, T.M., 2000, Methods of analysis by the U.S. Geological Survey National Water Quality Laboratory — processing, taxonomy, and quality control of benthic macroinvertebrate samples. U.S. Geological Survey Open-File Report 00-212 (IN PRESS).

12.2. National Research Council, 1995, Prudent practices in the laboratory—Handling and disposal of chemicals: Washington, D.C., National Academy Press, 427 p.

12.3. U.S. Geological Survey, 1999, Occupational Hazards and Safety Procedures Handbook: Manual No. 445-2-h, available at http://www.usgs.gov/usgs-manual/handbook/hb/445-2-h.html

13. Key Words

benthic macroinvertebrate, quantitative sample processing, fixed-count method

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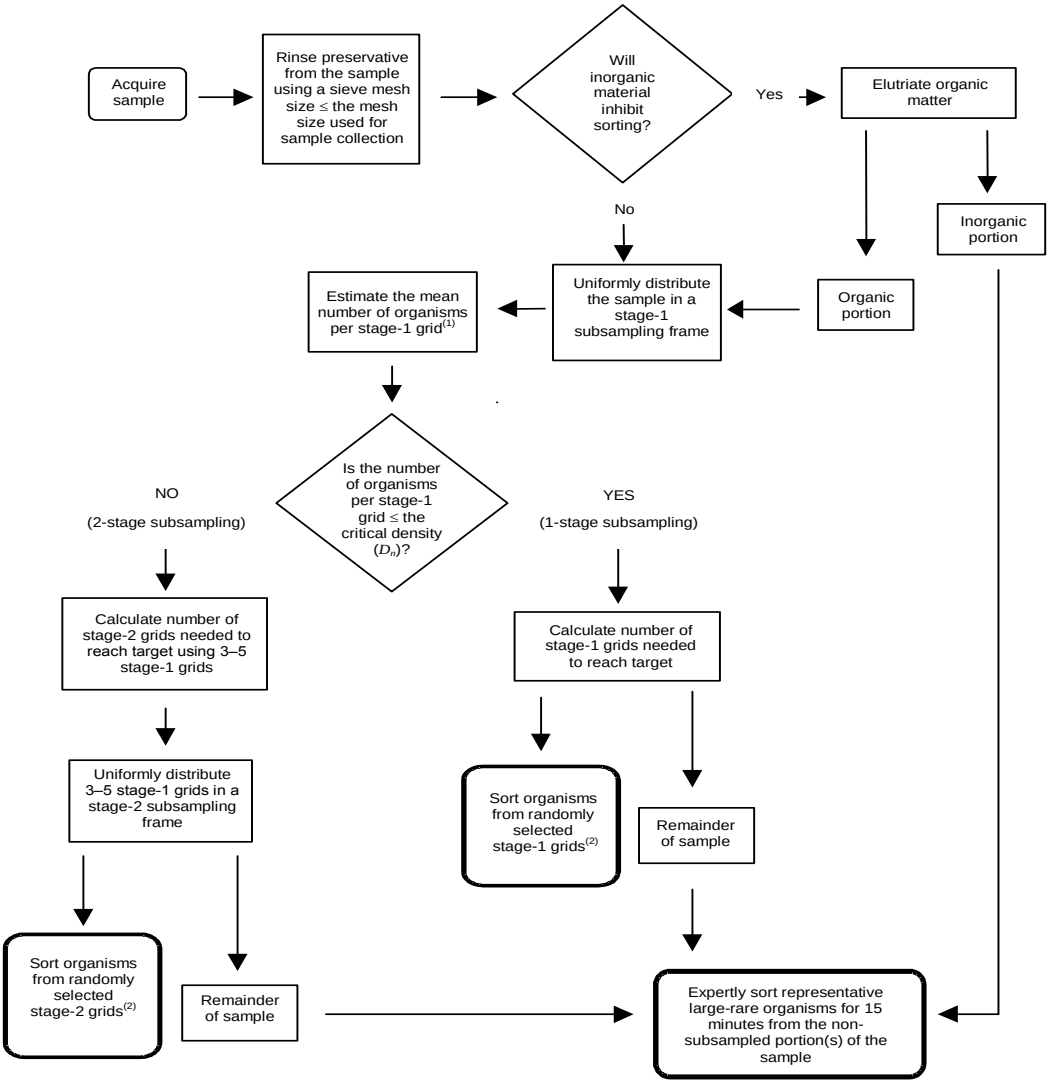
Appendix 1.— Overview of the quantitative, fixed-count method for processing benthic macroinvertebrate samples.



The mean number of organisms per subsampling frame is determined by using estimation trays that subsample each of five stage-1 grids.
At least 3 grids are always sorted. The maximum number of grids sorted is determined by numeric (fixed-count) and time criteria. Grids are sorted in their entirety until the fixed-count or processing time criteria are exceeded.

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Attachment

Quantitative BMI Sample Processing — Subsampling and Preliminary Enumeration Worksheet